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CD147 Triggers TCR Signaling Pathways by Activating JNK Signaling

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Abstract

T cell receptor signaling activation is important for proper T cell development and maintenance of effector T cell function upon suitable stimulation. We previously determined that CD147 (known as Basgin), a tumor-associated glycoprotein and a potential target for cancer immunotherapy, negatively regulates antitumor responses mediated by CD8+ TILs (Tumor-infiltrating lymphocytes). In this study, using genetic deletion, we showed that CD147 supports T cell receptor-mediated activation in both mouse and human T cells. Moreover, CD147-deficient T cells exhibits impairing T Cell Receptor (TCR)-related signaling events, including the phosphorylation of proximal TCR signaling molecules such as LCK and ZAP70 *in vitro* experiments. More importantly, CD147 is associated with TCR signaling and T cell proximal kinase activity by regulating the JNK signaling pathway. Altogether, we found a novel function of CD147 in T lymphocyte activation mediated by TCR proximal signaling and is a potential therapeutic target for preventing TCR-mediated T cell activation.

Keywords: CD147; T cell receptor; JNK signaling; Antitumor response; T cell exhaustion

Introduction

Normal T cell activation is critical for developing adaptive immunity and maintaining self-tolerance in the human body. T Cell Receptors (TCRs) expressed on T cells bind with Major Histocompatibility Complex (MHC)-antigenic peptides, activating downstream signaling and effector functions in T cells, such as cytotoxicity and cytokine secretion [1,2]. Early T cell proximal signaling of TCRs mainly includes the engagement and activation of various protein-tyrosine kinases, such as LCK, FYN, and ZAP70, which are key signaling pathway molecules in TCR signaling pathway activation [1,3,4]. LCK is the earliest known key proximal kinase that can induce ITAM phosphorylation, interacting with ZAP70 to activate ZAP70 kinase phosphorylation level and induce downstream signaling transduction [2,4,5]. Generally, intracellular signaling pathways regulate cellular fates and functional outcomes [6]. Members of the MAPK signaling pathway, such as p38, and JNK family proteins, play a vital role in the proliferation, differentiation, and function of different subsets of immune cells [2,7]. Some studies reported that insufficient JNK activity decreases the activity of transcription factors, including AP-1, and inhibits TCR activation signaling [8]. CD147, also known as EMMPRIN, is a trans-membrane glycoprotein that regulates T cell maturation, adhesion, and activation [9,10]. CD147 also binds to plasma membrane calcium ATPase isoform 4 (PMCA4) to reduce IL-2 cytokine production and bypass TCR proximal signaling in Jurkat T cells and primary $CD4^+$ T cells [11]. Some studies showed that CD147 could induce the dissociation of its coreceptors, such as CD48, from microdomains to regulate T cell receptor signaling in peripheral blood mononuclear cells [12,13]. Moreover, CD147 is highly associated with the maturation and circulation of immature thymocytes [14]. In the human immune system, CD147 deficiency in Tm cells greatly inhibits Tm cell activation [10,15]. Further studies demonstrated that the anti-CD147 mAb 5A12 decreases tyrosine phosphorylation and intracellular calcium mobilization levels in T cells to negatively regulate T cell activation by forming immune synapses [16]. However, to date, the exact mechanism of CD147 in regulating T cell activation remains unclear.

Here, we show that CD147 is highly expressed in the activated T cells and Jurkat cell lines. Furthermore, we also found that CD147 on the T cell membrane positively regulates TCR signaling pathways and induces T cell activation. Additionally, RNA sequencing and western blotting reveal that activating JNK signaling in Jurkat T cells is important in influencing T cell activation. Overall, our observations suggest that CD147 might positively influence TCR signaling pathways and

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induce T cell activation by regulating JNK signaling activation *via* phosphorylation.

Materials and Methods

Cell lines

Jurkat and ShCD147 Jurkat cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The culture medium of these cells was RPMI 1640 medium containing 10% FBS (Invitrogen) and penicillin and streptomycin (100 U/mL) (Invitrogen).

Animals

All animal protocols were approved by the Animal Care and Welfare Committee of the Fourth Military Medical University. C57BL/6 mice (6 to 8 weeks old) purchased from the Laboratory Animal Center of the Fourth Military Medical University were used in this study. $Bsg^{\Delta T}$ (Lck-cre^{+}, $Bsg^{flox/flox}$) and Bsg^{WT} (Lck-cre^{-}, $Bsg^{flox/flox}$) mice which were generated previously in our laboratory with a C57BL/6 background. Only paired littermates from the same breeding colony were used for the experiments. Mice were treated using protocols approved by the Laboratory Animal Ethics Committee of Fourth Military Medical University and all experiments were performed in accordance with national ethical guidelines as defined by the relevant national animal welfare. The experimental study was approved by Institutional Animal Care and Use Committee Review (2023-NTSCMM-ID004).

Isolation and culture of CD8^{+} T cells

Naïve CD8^{+} T cells were isolated using an EasySep™ Mouse naïve CD8^{+} T Cell Isolation Kit (19858, STEMCELL Technologies) from a mouse spleen cell suspension. To prepare mice splenic lymphocytes, spleen cells were prepared by gently crushing spleen tissues to release the cells. Following isolation, the cells were resuspended at a density of 1×10^{6} cells/mL in a T cell culture medium, which contains RPMI 1640 (Thermo 11875093), 10% Fetal Bovine Serum (FBS), 1 × Glutamax (Life Technologies #35050–061), 1 mM sodium pyruvate, 0.1% β-mercaptoethanol, and penicillin/streptomycin (10000 U/ml). The cells were then stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) antibodies in 96-well plates. In some experiments, the cells were also treated with 200 U/mL of mouse IL-2 (Peprotech, 212-12-20UG) for 72 h.

Retroviral transduction of Jurkat cells

Human CD147-specific shRNA-encoding retroviruses and negative control retroviruses were obtained from GenePharma (Shanghai, China). Jurkat cells were transfected using Lipofectamine 2000 reagent (Invitrogen, USA) with supernatant containing lentivirus carrying the shCD147 to generate CD147-knockdown cell lines, named shCD147 Jurkat cell lines. After 48 h, the infected cells were selected with 3 μg/mL puromycin and a monoclonal cell was selected for further study. The expression of CD147 in Jurkat cells was detected using western blot and flow cytometry to determine the silencing effects.

RNA isolation and real-time PCR analysis

To detect the expression of CD147, total RNA was collected from the cells using a Total RNA Kit II (Omega Bio-Tek, USA) and then reversely transcribed to cDNA using a PrimeScript RT Reagent Kit (TaKaRa, Kusatsu, Japan), according to the respective manufacturer's instructions. qPCR was carried out using a SYBR Premix Ex Taq II Kit (TaKaRa) on an Agilent Mx3005P, and data were analyzed with MxPro-Mx3005P software. The primer sequences used were as follows (Table 1):

Table 1: Primer sequences.

H-BSG-Forward-primer	ACTCCTCACCTGCTCCTTGA
H-BSG- Reverse -primer	GCCTCCATGTTCAGGTTCTC
H-CD69-Forward-primer	TCTTGTTCTGAAGATGCTGCTA
H-CD69-Reverse-primer	CTTCATTTTTCAGCCCAATCCA
H-TNF-α- Forward-primer	ACCTGAACGTCTCTTCCTCCCAAG
H-TNF-α- Reverse-primer	GGCAGCAGAACCAGCAGCAG
H-IL-2-Forward -primer	CACCAGGATGCTCACATTTAAG
H-IL-2-Reverse-primer	CTCCAGAGGTTTGAGTTCTTCT
H-GADPH-Forward -primer	GCACCGTCAAGGCTGAGAAC
H-GADPH-Reverse-primer	TGGTGAAGACGCCAGTGGA

Co-Immunoprecipitation (Co-IP) assay

The Co-IP assay was performed using a Pierce® Co-Immunoprecipitation Kit (26149, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Mouse anti-human CD147 antibody was coupled to the resin for immobilization in the Co-IP assay. The antibody-agarose beads were mixed with the lysate, and the mixture was incubated with gentle mixing at 4°C overnight. After washing with an ice-cold washing buffer six times, the eluted proteins were analyzed *via* Western blotting.

Western blotting

Equal amounts of proteins were loaded onto 10% or 12% SDS-PAGE gels and then transferred to PVDF membranes (Millipore, Boston, USA). After blocking with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h, the membranes were incubated with the corresponding primary antibodies at 4°C overnight. Images were developed after incubation with the corresponding secondary antibodies at room temperature for 1 h. Immunoblots were developed using an ECL kit (Beyotime Institute of Biotechnology, Jiangsu, China). The protein expression of each sample was quantified using ImageJ software (https://imagej.net/software/imagej), then the phosphorylation level of the LCK and ZAP70 phosphorylated proteins was measured by LCK and ZAP70 phosphorylated proteins expression was divided by the corresponding LCK and ZAP70 own proteins.

Antibody peptides and SP600125 stimulation experiments *in vitro*

The Jurkat cells are exposed in the growth cell medium which exists extremely addition and stimulation of CD147 antibody peptides (Produced from in our own laboratory) *in vitro* with different concentrations such as 0, 30 and 50 ng/ml for one day. The control experimental group are should applied on the normal growth cell culture without additional other reagents. For the SP600125 stimulation assay, we seeded cells at 6-well plates (Corning, Corning, NY, USA) with 2 ml cell culture medium/well. SP600125, an orally active, reversible, and ATP-competitive JNK inhibitor, inhibits autophagy and activates apoptosis. After incubation with SP600125 (MedChemexpress, HY-12041, USA) for 24 h in the wells with culture condition at 37°C in an atmosphere of 5% CO_2. After that, Western blotting experiment is performed.

Flow cytometry analysis

Related live cell samples are harvested for flow cytometry.

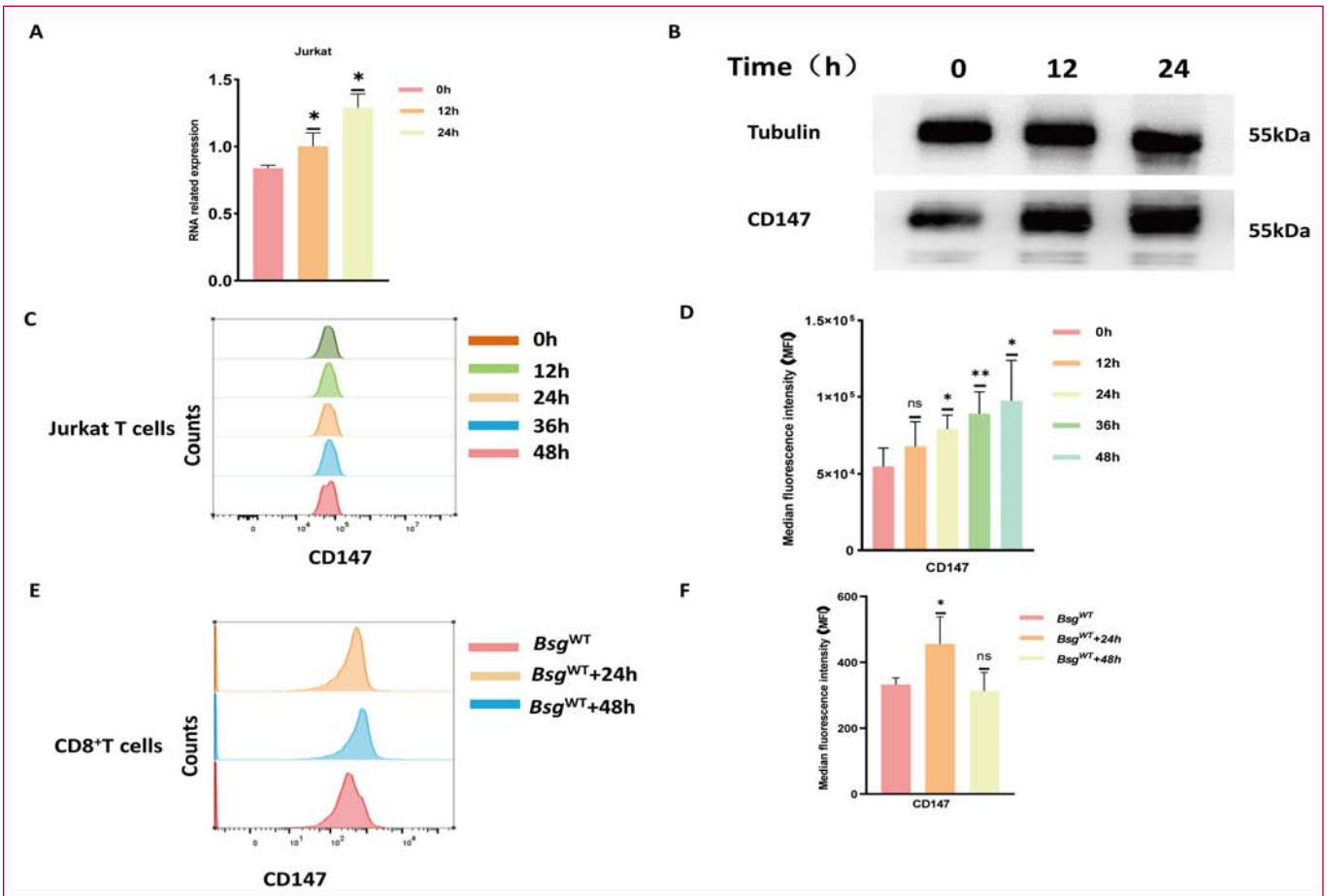

Figure 1: A, B) QPCR and western blotting analysis of CD147 expression in Jurkat cell lines activated for the indicated times (0, 12, and 24 h). C) Flow cytometry analysis to evaluate the expression level of surface molecules CD69, CD25, and CD147 in the corresponding groups of cells activated at different durations. D) Representative flow cytometry histograms depicting mouse CD69, CD25, and CD147 surface expression in the CD8+ T cells treated as indicated in the figure. Data are shown as relative mRNA level, which was calculated using the $2^{-\Delta\Delta CT}$ method and represented as the mean and Standard Error of the Mean (SEM). The p-values were determined using a two-tailed Student's t-test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns: not significant.

Cells were incubated with the following fluorochrome-conjugated antibodies 30 min in dark: FITC-conjugated anti-mouse CD8a (100706), PerCP-conjugated anti-mouse CD25 (102028, APC-conjugated anti-mouse CD69 (104514), APC-conjugated anti-human CD25 (356110), PerCP/Cyanine5.5-conjugated anti-human CD69 (310926), and APC-conjugated anti-human CD147 (306214) (all from Biolegend). Two million events were obtained for flow cytometry analysis using a FACSCalibur flow cytometer (BD, USA). FACS data were analyzed using FlowJo software (Tree Star Inc.). Specially, the MFI (Median Fluorescence Intensity) of each group is measured by the median in the FlowJo software and the histogram analysis was made in the Prism.

RNA Sequencing

Total RNA was extracted using a TRizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was qualified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked using RNase-free agarose gel electrophoresis. After total RNA was extracted, eukaryotic mRNA was enriched using Oligo(dT) beads. The enriched mRNA was sheared into short fragments using a fragmentation buffer and reverse-transcribed into cDNA using random primers. Second-strand cDNA was synthesized using a reaction system containing DNA polymerase I, RNase H, dNTPs, and a suitable reaction buffer. Then, the cDNA fragments were purified with a QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), end-repaired, poly(A) tails were added, and ligated to Illumina sequencing adapters. The ligation products were size-selected using agarose gel electrophoresis, PCR-amplified, and sequenced using an Illumina NovaSeq 6000 platform by Gene Denovo Biotechnology Co. (Guangzhou, China).

Immunofluorescence staining

Jurkat cells were harvested and allowed to attach for 24 h to cell culture dishes with glass bottoms (NEST, Wuxi, China) by the poly-L-lysine (Sigma-Aldrich, P4832). After being washed twice with PBS, the cells were fixed in paraformaldehyde in PBS, permeabilized with 0.3% Triton X-100, and blocked with 1% bovine serum albumin in PBS for 1 h. The cells were then incubated with the indicated primary antibodies for 1 h, washed twice with PBS, and incubated with the appropriate secondary antibodies according to the manufacturer's instructions. Cell nuclei were stained with DAPI for 10 min (Beyotime, C1006). After washing, the cells were visualized using an A1R-A1 confocal laser microscope system (Lecia).

Results

CD147 mRNA and protein are highly expressed in activated T cells

To elucidate the expression status of CD147 in T cell activation, we analyzed the mRNA and protein expression level of CD147 in Jurkat T cell lines at different activation times and using CD3 and

CD28 antibodies (Figure 1A, 1B). These figures show that CD147 was highly expressed in the activated Jurkat T cell group *versus* the control group. We then continued to prolong CD3 and CD28 activation time to 36 h and 48 h and examined the expression level of CD147 *via* flow cytometry. The results show that CD147 expression was consistently up regulated in the indicated activated times (Figure 1C, 1D). Furthermore, we also used a C57BL/6 mouse model to isolate splenic $CD8^+$ T cells *in vitro* to identify CD147 expression levels. The results demonstrated that CD147 expression also increased in activated $CD8^+$ T cells with specific time 24 h, compared to naïve T cells (Figure 1E, 1F). Thus, the surface marker CD147 was unregulated in the activated T cells and may regulate T cell activation.

CD147 knockout reduces the number of $CD69^+$ T cells in the spleen and impairs the TCR proximal signaling pathways

To determine the role of CD147 in the subtypes of early activated T cells, we analyzed the populations of the $CD69^+$ $CD8^+$ and $CD69^+$ $CD4^+$ T cells in the CD147-specific knockout mice compared with wild-type mice. The results showed that CD147 knockout substantially reduced the population of activated $CD69^+$ $CD8^+$ and $CD69^+$ $CD4^+$ T cells (Figure 2A-2C). To evaluate if CD147 may influence T cell apoptosis, we assessed the number of apoptotic cells in the Jurkat T cell line followed by Annexin V and 7-AAD staining. We found that CD147 deficiency did not affect apoptosis in T cells (Figure 2D, 2E). To investigate if the influence of CD147 on T cell activation involved regulating TCR proximal signaling, we evaluated the TCR proximal kinase phosphorylation levels in the ShCD147 Jurkat cell and control groups. The results suggest that LCK phosphorylation at Tyr505 was increased and ZAP70 phosphorylation at Tyr319 and LCK Tyr394 phosphorylation was decreased compared to ShCD147 Jurkat cells, suggesting CD147 knockout in the T cell decreased TCR signal transduction (Figure 2F, 2G and Supplementary Figure 1, 2). Thus, we confirmed that CD147 knockout in T cells reduce activated T cell amounts in the spleen and negatively regulates T cell activation by impairing the TCR signaling pathway.

CD147 antibody peptides contact membrane CD147 *in vitro* and enhance TCR proximal signaling

We also determined whether CD147 regulates the TCR signaling pathway in a specific manner. Optimal TCR signaling transduction requires some molecules expressed in the tumor cell and in the TME

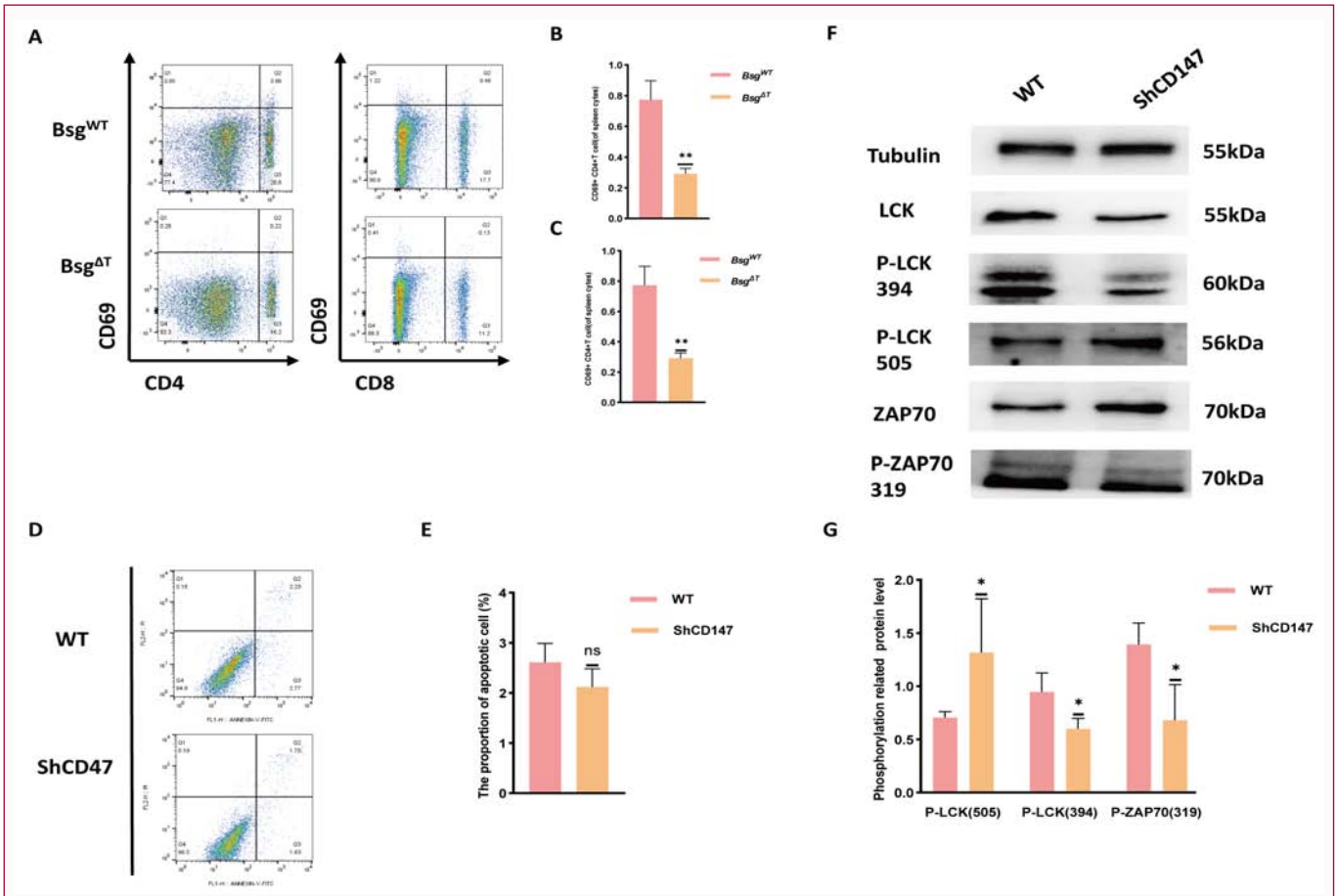

Figure 2: A) The frequencies of CD69+ CD8+ and CD69+ CD4+ T cells in the total spleen T cell population from BsgWT or BsgΔT mice were analyzed using flow cytometry (four pairs of mice). B) The frequencies of apoptotic cell in the CD147-KO Jurkat cell and control groups was analyzed by Annexin V/PI flow cytometry staining. C) Western blotting was performed to detect LCK and ZAP70 phosphorylation levels in the NC and CD147-KO Jurkat cell lines. D) LCK and ZAP70 phosphorylation level was normalized relative to LCK and ZAP70 protein expression. Statistical histogram of LCK and ZAP70 phosphorylation generated using Prism 9.0 software. E) Representative flow cytometry result demonstrating CD69 and CD25 expression and the phosphorylation level of LCK at Tyr505 and ZAP70 at Tyr319 on corresponding Jurkat cell lines treated with anti-CD3 and anti-CD28 antibodies or non-TCR-based stimulations. F) Representative flow cytometry result showing CD69 and CD25 expression on corresponding CD8+ T cell lines fractions in the splenic T cells from BsgWT or BsgΔT mice treated with anti-CD3 and anti-CD28 antibodies *in vitro*. The numbers in or adjacent to outlined areas (or in quadrants) indicate percentages. Each symbol represents an individual mouse. Summary graphs are presented as the mean ± Standard Error of the Mean (SEM) (n=6 mice per group). P-values were determined using a two-tailed Student's t-test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns: not significant.

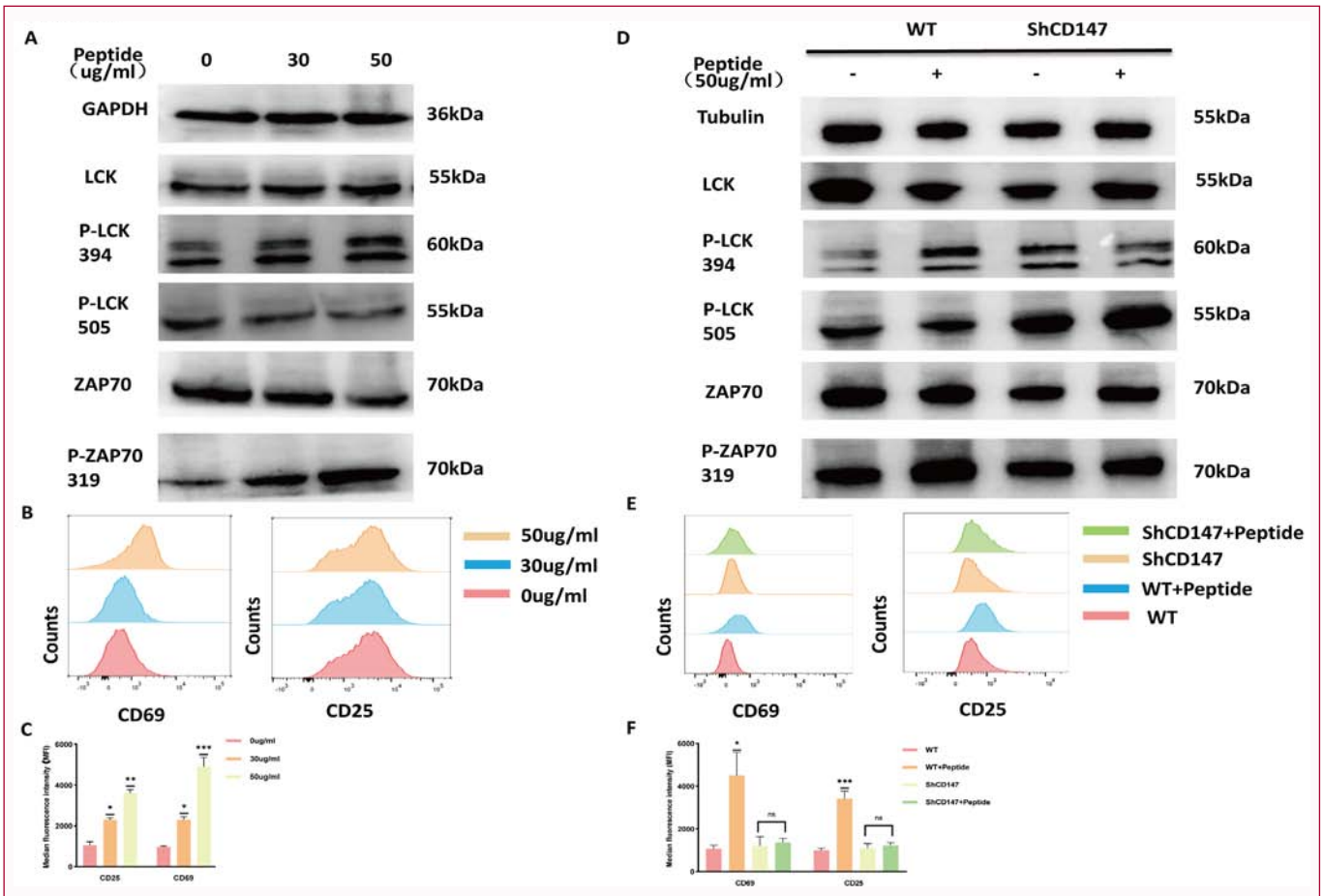

Figure 3: A) The TISCH database, a comprehensive web resource enabling interactive single-cell transcriptome visualization of the Tumor Microenvironment (TME), was used to determine the relationship and profiling of CD147 expression and cell clustering of exhausted T cell subtypes in the TME, specifically in the NSCLC (Non-Small Cell Lung Carcinoma) randomly according to the 4 different NSCLC patients' samples. B) Flow cytometry histogram showing the differences in TNF-α, granzyme-B, and IFN-γ expression in Jurkat cells compared to CD147-KO cells after activation for one day. C) QPCR analysis of IL-2 and TNF-α expression in the Jurkat and CD147-KO groups in the indicated activated time (24 h). Data are shown as relative mRNA levels calculated using the $2^{-\Delta\Delta CT}$ method and represented as the mean and Standard Error of the Mean (SEM). The p-values were determined using a two-tailed Student's t-test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns: not significant.

via a collaborative connection, such as costimulatory B7-1/B7-2 and adhesion molecule receptors [3,5]. Therefore, we exploited *in vitro* CD147, which is mainly derived from tumor cells in the TME, to explore whether the interaction isotype in the T cell surface strengthens TCR signaling and facilitates T cell activation. We exposed Jurkat cell lines on different concentrations of CD147 antibody peptides *in vitro* and analyzed the phosphorylation levels of TCR proximal kinases, such as LCK-Tyr505/Tyr394, Zap70-Tyr319. The phosphorylation of TCR proximal kinase active sites ZAP70-Tyr319 and LCK-Tyr394 was also increased; in contrast, phosphorylation of the inactive site LCK-Tyr505 was decreased with T cell cultured with higher antibody peptides (Figure 3A). We found that CD69 and CD25 expression was increased in a CD147 antibody peptides concentration-dependent manner (Figure 3B, 3C). This finding indicates that CD147 antibody peptides positively influence the TCR signaling pathway *in vitro*. Next, we evaluated whether CD147 antibody peptides act on the TCR signaling by binding to CD147 on T cells. We separately added the maximum concentration of CD147 peptides on the wild-type and ShCD147 Jurkat cells. The results showed that CD147 peptides induced higher CD69 and CD25 expression in the wild-type Jurkat cell lines and positively regulated TCR proximal kinase activation levels compared to the ShCD147 Jurkat cell group. In contrast, CD147 peptides have no clear influence on T cell activation in ShCD147 Jurkat cells (Figures 3D-3F). Collectively, these results indicate that CD147 antibody peptides enhance TCR proximal signaling and positively regulate TCR proximal kinase activity by binding to CD147 on T cells.

CD147 induced T cell activation may *via* the JNK signaling pathway

To further elucidate the mechanism of how CD147 regulates the TCR signaling pathway, we analyzed the RNA sequences of ShCD147 Jurkat and control cell lines. In total, we identified 294 upregulated and 671 downregulated genes between cluster 1 and cluster 2 (Figure 4A). We analyzed the top 30 differentially expressed genes. ASS1, SLC385C, and ALDOC are related to cell metabolism; PTPRK, DOCK2, and PRDX1 are abundantly expressed in tumor cells, which mainly influence cell activation and cellular stress. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis for differential genes was also performed (Figure 4B, 4C). The signaling pathways include AMPK signaling, P13-AKT signaling, and transcriptional mis regulation in cancer signaling. Overall, the RNA-seq data support the view that ShCD147 Jurkat cells governs cell metabolism and activation mainly through the MAPK signaling pathway. Furthermore, T cell activation is one of the most vital characteristics of T cell-mediated tumor cell killing.

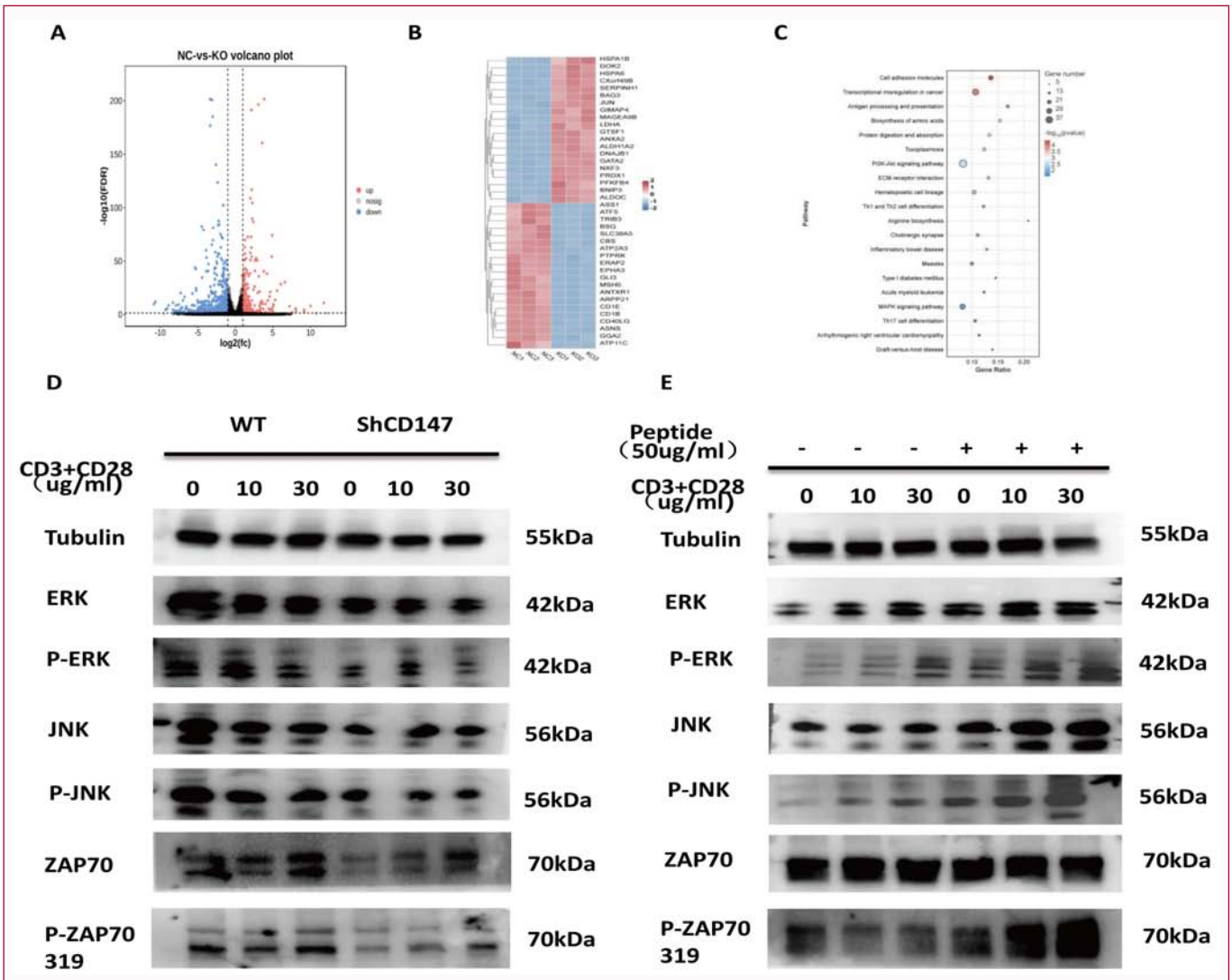

Figure 4: A) Western blotting was used to detect T Cell Receptor (TCR) kinase activity and LCK and ZAP70 phosphorylation protein levels in Jurkat cell lines treated with different doses of CD147 antibody peptides (0, 30, and 50 ng/mL). LCK and ZAP70 phosphorylation level was normalized to total LCK and ZAP70 protein expression B) Flow cytometry histogram demonstrating CD69 and CD25 expression in Jurkat cell lines treated with the indicated concentrations of CD147 antibody peptides. C) Flow cytometry histogram demonstrating CD69 and CD25 expression in Jurkat and CD147-KO Jurkat cells before and after adding CD147 antibody peptides. D) Western blotting was used to detect LCK and ZAP70 phosphorylation levels in the same experimental groups. The data are presented as the mean ± SEM from at least three independent experiments. *p<0.05; **p<0.01, ***p<0.001 (Mann-Whitney or ANOVA with Tukey's post-test analysis).

MAPK signaling pathway activation is a key regulator that affects T cell activation and related KEGG pathways, according to the RNA-seq data. Thus, we tried to determine whether CD147 regulates T cell activation through the MAPK signaling pathway. Based on the previous results, numerous studies have demonstrated that CD147 regulates ERK signaling in the T cells. We first clarified that CD147 whether can regulates JNK signaling in the T cells. We analyzed the phosphorylation level of key kinases in the MAPK pathway in the CD147-KO Jurkat cell and control groups. The depletion of CD147 decreased the JNK pathway-mediated phosphorylation of JNK and ERK and inhibited TCR proximal kinase activation in ZAP70-Tyr 319 (Figure 4D). And we also added CD147-specific peptides in Jurkat cells with related maximum activated intensity (Figure 4E), we also founded that CD147 peptides additions *in vitro* can increases JNK signaling pathway transduction. All and all, we confirm that CD147 knockout in the T cells negatively influences JNK signaling and represses T cell proximal kinases ZAP70 phosphorylation level.

SP600125 can negatively regulates CD69 and CD25 expression in Jurkat and Splenic CD8⁺ T cells *in vitro*

SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase, is often to block JNK phosphorylation [19,20]. To elucidate whether JNK signaling influences T cell activation, we examined the changes in the expression of CD69 and CD25 in Jurkat and $CD8^+$ T cells. The data demonstrated that SP600125 treatment significantly decreased CD69 and CD25 expression in Jurkat on a concentration-dependent manner (Figure 5A and 5B). And this experimental protocol was applied to mouse $CD8^+$ T cells, yielding the same conclusion (Figure 5C, 5D). Additionally, the phosphorylation of LCK and ZAP70 also decreased after treatment with SP600125 (Figure 5E). The relative RNA level of CD147 and CD69 also decreased in the Jurkat cell lines in a SP600125 concentration-dependent manner (Figure 5F). These results suggest that the JNK signaling pathway plays a role in T cell activation and that additional JNK signaling key molecules phosphorylation reinforces stronger T cell activation. Thus, we concluded that the JNK signaling pathway positively induces T

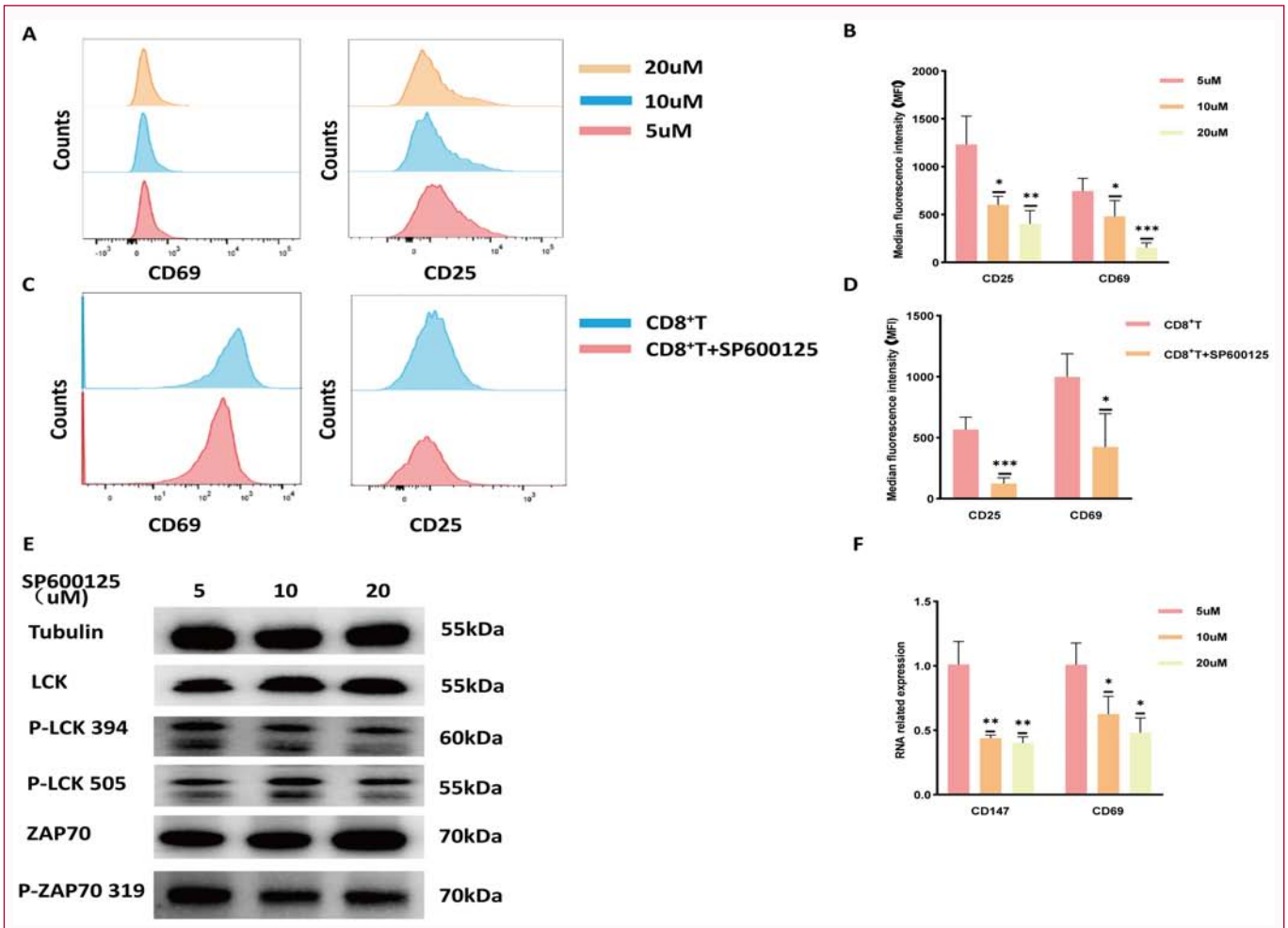

Figure 5: A) Volcano plot of NC *vs.* CD147-KO Jurkat cells is shown in the RNA-seq data. Blue dots represent downregulated proteins, red dots represent upregulated proteins, and gray dots showed proteins with no statistically significant difference between the NC and CD147-KO Jurkat cell groups. B) The top 30 differentially expressed genes are shown. Red represents upregulated genes and blue represents downregulated genes compared to NC Jurkat T cells. C) KEGG pathway analysis among the NC and CD147-KO Jurkat cell groups. D) Western blotting analysis of the JNK signaling pathway in the CD147-KO and NC Jurkat cell groups independently cultured on plates previously coated with anti-CD3 and anti-CD28 antibodies at the indicated concentrations. Western blotting images show the expression of JNK signaling pathway-related proteins. GADPH was used as a loading control. E) Western blotting analysis of JNK signaling pathway-related proteins in Jurkat cells in plates previously coated with anti-CD3 and anti-CD28 antibodies in the indicated concentrations and incubated with CD147 peptides at different concentrations (0, 30, and 50 μg/mL) for one day. GADPH was used as a loading control. The data represent the mean ± SEM from at least three independent experiments. Two-way Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

cell activation in Jurkat and CD8⁺ T cells *in vitro*.

CD147 disconnects CD4 and CD8 in Jurkat T cell and relates T cell exhaustion

The process of T cell receptor activation involves multiple transduction pathways [1,5,21]. CD4 and CD8, which are expressed in the T cell membrane and regulate the coupling of the T cell receptor complex (CD3-TCR), play crucial functions in T cell activation [5,21]. Therefore, we detected whether CD147 is involved in the direct binding of CD4 and CD8 to influence T cell proximal activation. Co-IP experiments demonstrated that CD147 could dissociate from CD4 and CD8 (Figure 6A, 7C). And the results of immunofluorescence staining demonstrates that CD147 don't colocalizes with CD4, and CD8 in the Jurkat cells (Figure 7B, 7D). Thus, these results show that CD147 regulates T cell activation and TCR proximal kinases activity without *via* binding with CD4 and CD8 molecules. It was reported that CD147 is involved in regulating the phosphorylation of the TCR proximal kinase; however, it plays an immunosuppressive role in the T cell-mediated immune response [17,18]. Thus, we speculated that CD147 may be associated with exhausted T cell subtypes. We used the TISCH (Tumor Immune Single Cell Hub) database to evaluate the correlation between CD47 and exhausted T cells in the tumor microenvironment. A close relation was found between CD147 expression level and immune infiltration in non-small cell lung cancer, skin cutaneous melanoma, and breast cancer (Figure 6E).

Discussion

In the present study, we demonstrate a previously unrecognized mechanism by which CD147 regulates the activity of TCRs in the cell surface to influence T cell activation. CD147 expression is upregulated following T cell activation [22]. Here, we further identify that CD147 deficiency dampened T cell activity and decreased the activity of proximal kinases such as LCK and ZAP70 following TCR activation, and the expression of T cell activation surface markers such as CD69 and CD25 in Jurkat cells and mouse spleen-derived T cells. Moreover, CD147 deficiency decreased CD69 and CD25 expression in the CD8⁺ T cells *in vivo*. Thus, CD147 might play an important role in TCR-CD3 receptor activation signaling modulation. Previous studies

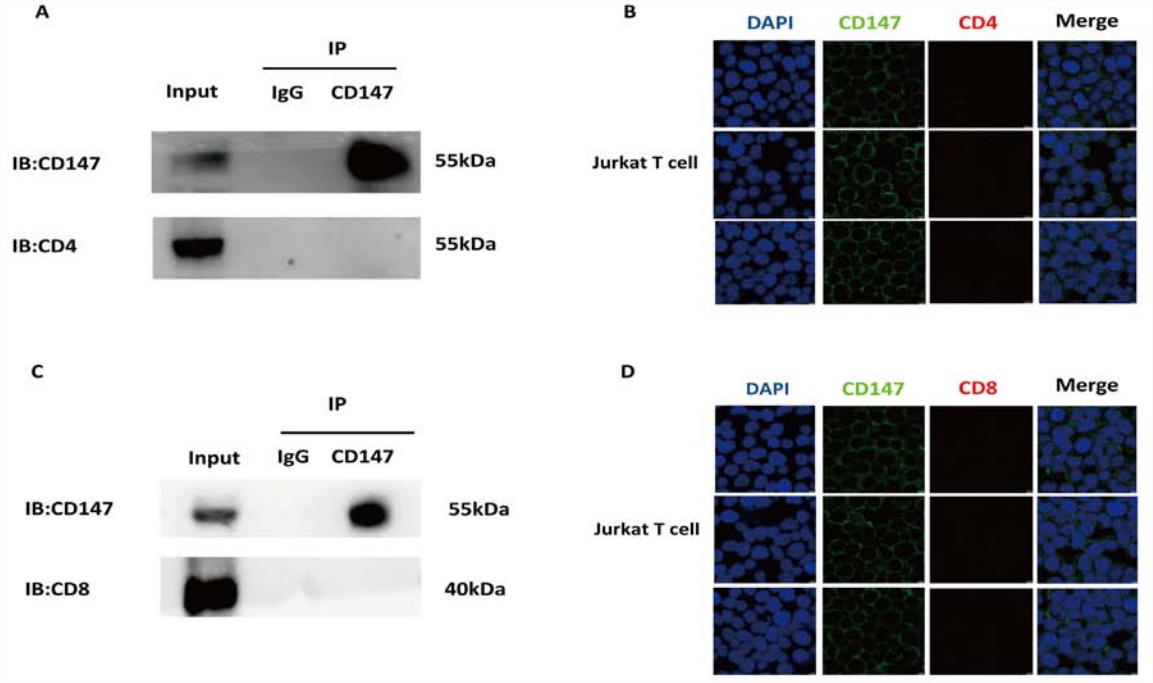

Figure 6: A) Flow cytometry histogram demonstrating CD69 and CD25 expression in Jurkat cells treated with SP600125 in the indicated concentrations (5, 10, and 20 μM). B) QPCR analysis of the CD147 and CD25 expression level in Jurkat cell lines treated with SP600125 in the indicated concentrations (0, 10, and 20 μM) C) Western blotting analysis of TCR signaling pathway-related proteins such as LCK and ZAP70 phosphorylation level in Jurkat cells incubated with different concentrations of SP600125 *in vitro* for one day. GADPH was used as a loading control. D) Effects of in the presence or absence of SP600125 with different concertation on 5, 10, and 20 μM on gene expressions such as CD69 and CD147 in Jurkat cells lines. The target mRNA related expression was normalized to that of the internal control 18S rRNA. E) Flow cytometry histogram demonstrating CD69 and CD25 expression on the CD147-KO and NC Jurkat cells treated with SP600125. Data represent the mean ± SEM from at least 3 independent experiments. Two-way Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

suggest that CD147 is a negative regulator of antitumor responses mediated by $CD8^+$ TILs and identified CD147 as a potential target for cancer immunotherapy [17]. Herein, we show that CD147 positively regulated T cell activation; conversely, it negatively affected T cell-mediated tumor immunotherapy. Some recent studies showed that continuous antigen stimulation or over-activation may lead to T cell exhaustion [23,24]. We also used the TISCH database to demonstrate that CD147 is associated with T cell exhaustion. Therefore, it is of great importance and urgency to elucidate if CD147 is closely related to T cell exhaustion and could induce an exhausted T cell subtype.

RNA sequencing was performed to further assess the mechanism by which CD147 regulates TCR activity. Strikingly, the MAPK signaling pathway is one of the important differentially expressed signaling pathways in regulating cell metabolism and activation in ShCD147 Jurkat cells compared to the control group. Some previous studies also reported that the JNK pathway is closely related to T cell activation and is a typical pathway regulating immune T cell function and activation [25,26]. Thus, we explored whether CD147 could regulate T cell activation through the JNK signaling pathway. First, we determined the relationship between the JNK pathway and T cell activation using the JNK inhibitor SP600125. We showed that activating the JNK signaling pathway positively regulates CD69 and CD25 expression and induces T cell activation, consistent with previous studies [27,28]. Previous studies have shown that CD147 acts as a transmembrane glycoprotein from the Immunoglobulin Superfamily (IgSF) and regulates many physiological processes, including inflammatory responses, cell proliferation, differentiation, survival, and apoptosis. Hence, it could be a specific biomarker involved in tumorigenesis and cancer progression and a target for cancer immunotherapy for many classic pathways, such as the MAPK and NF-κB signaling pathways [9,18,29].

We further explored whether CD147 could regulate the JNK signaling pathway to influence T cell activation. We showed that CD147 induces JNK pathway transduction to regulate T cell activation. JNK activation is complex; it is activated by the upstream molecule MAPKK, and its family consists of three proteins: JNK1, JNK2, and JNK3 [30,31]. Different downstream proteins activate different downstream nuclear transcription factors to affect various cell functions [32]. In this study, we used various doses of SP600125 to repress the JNK signaling pathway. Thus, it is necessary to explore which specific JNK protein influences T cell activation. CD147 has a long intracellular domain that can bind with many other molecules to regulate cell function and differentiation [33]. The TCR, a multi-component signaling complex, includes other key components such as CD4, CD8, and CD45 [21,34]. These molecules are expressed in the T cell membrane and interact with other molecules to regulate T cell signal transduction. Thus, we further demonstrated that CD147 binds with CD4, CD8, and CD45 to influence T cell activation. Our data show that CD147 disconnects CD45, CD4, and CD8 in the T cell membrane.

Conclusion

In summary, our study revealed a novel role for CD147 as a positive regulator of T cell activation *in vivo* the JNK signaling pathway. Our experimental data provide a framework by which endogenous CD147 may regulate TCR systems to maintain homeostasis and, when dysregulated, contribute to the progression of diseases such as cancer.

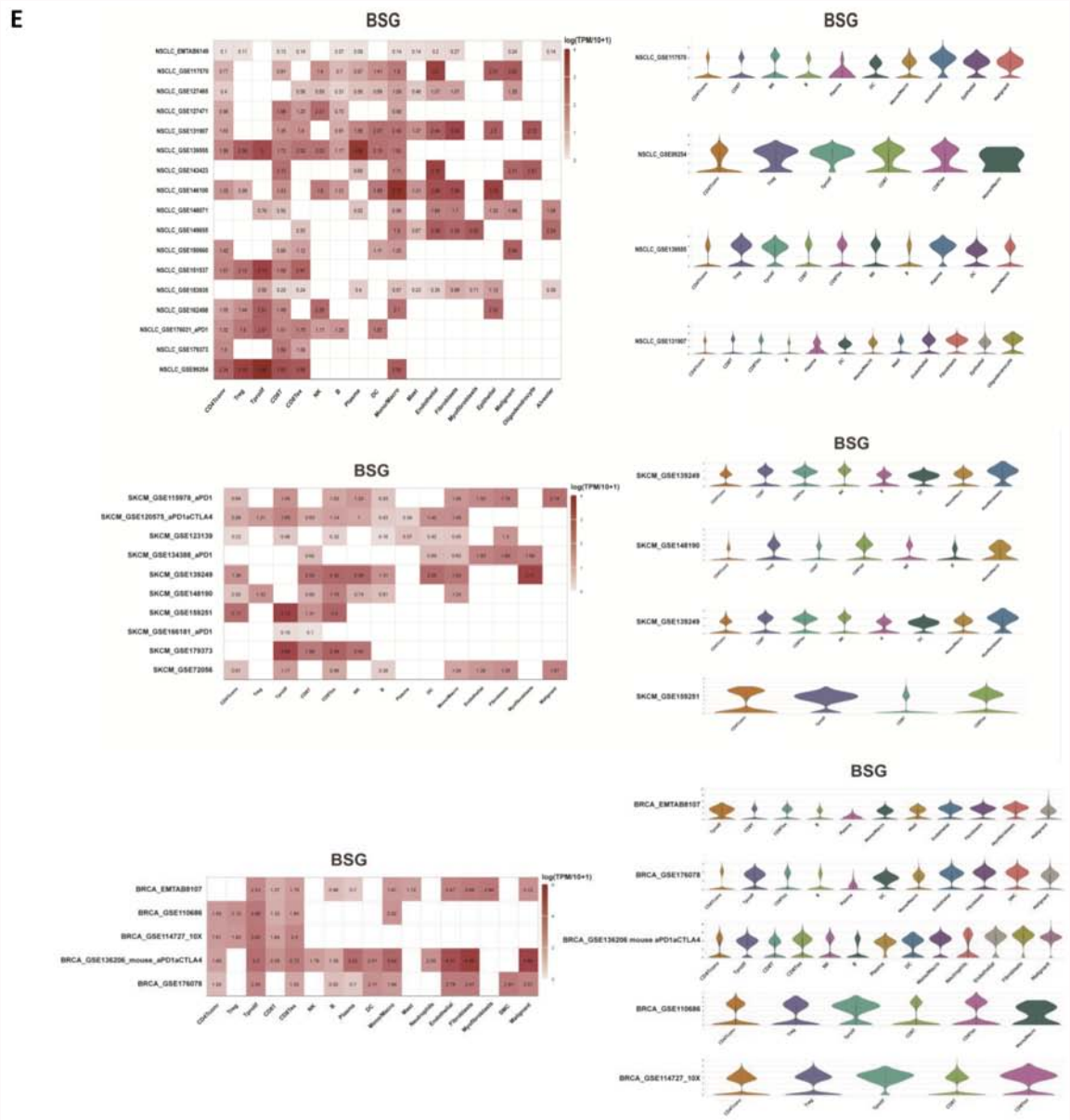

Figure 6: A) Flow cytometry histogram demonstrating CD69 and CD25 expression in Jurkat cells treated with SP600125 in the indicated concentrations (5, 10, and 20 μM). B) QPCR analysis of the CD147 and CD25 expression level in Jurkat cell lines treated with SP600125 in the indicated concentrations (0, 10, and 20 μM) C) Western blotting analysis of TCR signaling pathway-related proteins such as LCK and ZAP70 phosphorylation level in Jurkat cells incubated with different concentrations of SP600125 *in vitro* for one day. GADPH was used as a loading control. D) Effects of in the presence or absence of SP600125 with different concertation on 5, 10, and 20 μM on gene expressions such as CD69 and CD147 in Jurkat cells lines. The target mRNA related expression was normalized to that of the internal control 18S rRNA. E) Flow cytometry histogram demonstrating CD69 and CD25 expression on the CD147-KO and NC Jurkat cells treated with SP600125. Data represent the mean ± SEM from at least 3 independent experiments. Two-way Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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